The Thermodynamics of Formation of a Three-Strand, DNA Three-Way Junction Complex†

John E. Ladbury,*,‡ Julian M. Sturtevant,*,§ and Neocles B. Leontis*,‖

Departments of Chemistry and Molecular Biophysics and Biochemistry, Yale University, P.O. Box 208107, 225 Prospect Street, New Haven, Connecticut 06520, and Department of Chemistry, Bowling Green State University, Bowling Green, Ohio 43403

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ABSTRACT: Isothermal titration calorimetry (ITC) is used to study the thermodynamics of assembly of the three DNA oligonucleotides S1 (5′-GCCTGCCACCGC), S2 (5′-GCGGTGCGTCCG), and S3AA (5′-CGGACGAAGCAGGC) to form a three-way junction (TWJ) complex consisting of three double-helical arms radiating from a junction region having two unpaired adenosines in one strand (S3AA). The thermodynamics of assembly were measured for three different orders of addition of the component oligonucleotides at four temperatures between 10 and 25 °C. At each temperature studied, the overall values of ΔH, $\Delta S°$, and $\Delta G°$ for assembly of the complex from the component single strands were found to be independent of the order of addition. The enthalpy of binding, ΔH, was found to be linearly dependent on temperature. From the temperature dependence of ΔH, the change in heat capacity ΔC_p, for the overall assembly of three strands to form the junction complex was calculated and found to be −1.6 kcal mol^{-1} K^{-1}. This work represents the first attempt to evaluate the thermodynamics of DNA TWJ formation by ITC.

Three-way junctions (TWJs)[1] are the simplest multistem nucleic acid structures. They are comprised of three double-helical arms, which form when three mutually complementary nucleic acid strands converge in an antiparallel sense, as shown in Figure 1. Alternatively, they may result from the fusion of three nucleic acid duplexes. TWJs occur commonly as stable structural elements of biologically active nucleic acid molecules and as intermediates in nucleic acid metabolism. Examples include the 5S ribosomal RNA, found in the large ribosomal subunit (Noller, 1984), and the hammerhead ribozymes (Hutchins et al., 1986). The large 16S and 23S ribosomal RNAs (and their larger eukaryotic counterparts) all contain several TWJs (Noller, 1984). TWJs are likely to occur as structural elements in single-stranded DNA molecules, such as in the genomes of certain viruses. Besides their intrinsic interest, TWJs are also studied to gain further insight into other complex oligonucleotide structures, for example, the four-way junctions [reviewed by Lilley and Clegg (1993)].

Progress in understanding how nucleic acids carry out their functions depends in part on our ability to predict the secondary and tertiary structures of nucleic acids on the basis of their primary sequences. The best computer programs are least reliable when predicting the structures of nucleic acids containing multibranched loops (Jaeger et al., 1989; Zuker, 1989). DNA and RNA nucleic acid sequence data have accumulated rapidly in recent years, but progress in predicting the functional structures of nucleic acids has been hampered by the paucity of thermodynamic quantification and high-resolution structural data on higher order structures, such as three-way junctions (Turner & Sugimoto, 1988). Therefore, thermodynamic characterization of junction formation is crucial to further progress in predicting nucleic acid secondary and tertiary structure.

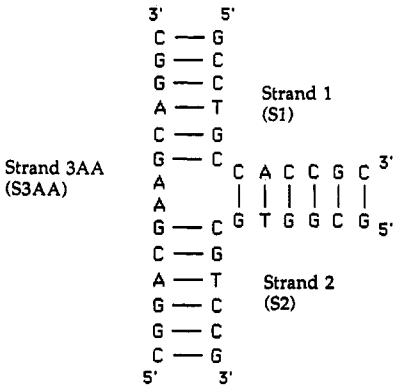

FIGURE 1: Schematic diagram of the S1:S2:S3AA DNA three-way junction (TWJ) that is the subject of this paper. The complex consists of three DNA oligonucleotide strands designed to hybridize with each other to form three double-helical arms. Strand 3AA contains two unpaired adenosines.

In this work, we use the TWJ shown in Figure 1 as a model for formation of this type of complex. Extensive NMR studies have been carried out on a very similar TWJ complex, S1:S2:S3TT, which differs from S1:S2:S3AA used in this study only in that it has two unpaired thymidines in the junction region instead of two adenosine nucleotides and it has only five base pairs per arm (Leontis, 1993). NMR spectra obtained on gradual heating of the S1:S2:S3TT TWJ showed that each arm was stable and fully base paired, forming normal B-type right-handed helices, up to at least 30 °C. The TWJ investigated in this work is likely to be more stable than that used for the NMR studies since it has one extra G-C base pair in each arm.

This is the first report of the use of high-sensitivity isothermal titration calorimetry (ITC) for determining the thermo-

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* Address correspondence to any author.

‡ Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115.

§ Yale University.

‖ Bowling Green State University.

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[1] Abbreviations: ITC, isothermal titration calorimetry; TWJ, three-way junction; NMR, nuclear magnetic resonance spectroscopy.

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dynamic parameters for DNA oligonucleotides forming TWJ systems and forms the basis for further research in this area.

MATERIALS AND METHODS

Nucleic Acid Synthesis and Purification. Studies based on melting curves have established thermodynamic parameters for DNA TWJs (with five base pairs in each arm; Leontis et al., 1991) and indicate that the formation of DNA TWJ complexes is stabilized by the inclusion at the branch point of two or more unpaired bases in one strand. Thus, in the work reported here, we incorporated two adenosine nucleotides on S3 (hence, S3AA).

Oligonucleotides were synthesized by the Midland Certified Reagent Company (Midland, TX) on a 10-μmol scale using phosphoramidate chemistry and were purified by preparative HPLC using ion-exchange chromatography (for oligos S1 and S2) or reverse-phase chromatography (for oligo S3AA). Oligonucleotide concentrations were quantified by UV absorbance using extinction coefficients calculated from published nearest neighbor parameters (Puglisi & Tinoco, 1989). The following molar extinction coefficients (260 nm) were calculated, for S1 (5′-GCCTGCCACCGC), 102 480; for S2 (5′-GCGGTGCGTCCG), 111 140; and for S3AA (5′-CGGACGAAGCAGGC), 143 340. Sample concentrations were independently checked by weighing the lyophilized oligonucleotides before dissolution in the sample buffer. The relative concentrations were further verified by mobility shift titration of pairs of oligonucleotides using native gel electrophoresis. The buffer used for dissolution of the oligonucleotides consisted of 0.10 M NaCl, 5 mM $MgCl_2$, 10 mM sodium phosphate, and 0.5 mM EDTA at pH 7.0. A divalent ion such as Mg^{2+} must be present for stable TWJ complexes to form at low to intermediate ionic strengths (Leontis et al., 1991).

Isothermal Titration Calorimetry (ITC). Experiments were carried out using the OMEGA isothermal titration calorimeter (MicroCal Inc., Northampton, MA). Data were analyzed using the ORIGIN software provided with the instrument. This instrument has been fully described by Wiseman et al. (1989).

Pairs of strands were first titrated, in the buffer solution described above, to form partial duplexes comprising one arm of the junction. After a given two-strand titration was completed, the solution in the cell was removed and thoroughly mixed, to include the fill-tube "dead volume" in the titration. The concentrations of the two oligonucleotides were equalized by addition of a sufficient amount of the strand in deficit. The two-strand complexes were returned to the calorimeter cell and titrated by addition of the appropriate third strand to form the TWJ complex. Heats of dilution for each oligonucleotide solution injected into buffer and buffer into the oligonucleotide(s) in the cell were measured separately and used to correct the data.

For all the reactions, the concentration of the oligonucleotide in the syringe was between 9 and 49 times greater than that in the ITC reaction cell. The oligonucleotide solutions were injected from either a 100- or 250-μL capacity syringe, maintained at constant temperature for the duration of the experiment, into the 1.39-mL isothermal cell containing the reactant solution.

The titration curve, corrected for dilution effects, was fit to obtain the binding constant, K_B, for the interaction as well as the stoichiometry, N, and the molar enthalpy of reaction, ΔH [as has been described by Wiseman et al. (1989)]. From these quantities, the molar free energy, $\Delta G°$, and entropy, $\Delta S°$, may be calculated using the relationships $\Delta G° = -RT \ln K_B$ and $\Delta G° = \Delta H - T\Delta S°$. The individual titrations were performed at a fixed temperature maintained by the calorimeter. Therefore, performing a series of reactions at different temperatures allows the temperature dependence of the enthalpy or heat capacity change, ΔC_p, to be determined.

The thermodynamic parameters for TWJ formation were determined in the ITC by, first, reacting two strands and subsequently adding the third strand. In the two-strand titrations, one arm of the junction was formed, as shown in eqs 1–3. The following nomenclature will be used to define the reactions concisely. A reaction in which strand 2 (S2), for example, was placed in the ITC reaction cell and strand 1 (S1) was added to it is represented by S2:S1. The further titration of this complex with strand 3AA (S3AA) is represented by S2:S1:S3AA. Equations 1–3 represent the two-strand titrations that were carried out.

$$S1 + S2 = S2{:}S1 \quad (1)$$

$$S3AA + S2 = S2{:}S3AA \quad (2)$$

$$S1 + S3AA = S3AA{:}S1 \quad (3a)$$

$$S3AA + S1 = S1{:}S3AA \quad (3b)$$

To assess whether the order of addition of the strands had any effect on the measured heats, the reaction of eq 3a was performed in reverse as represented by eq 3b.

The third strand was added to each two-strand complex to give the TWJ final product. Equations 4–6 represent the three-strand titrations that were carried out:

$$S3AA + S2{:}S1 = S2{:}S1{:}S3AA \quad (4)$$

$$S1 + S2{:}S3AA = S2{:}S3AA{:}S1 \quad (5)$$

$$S2 + S3AA{:}S1 = S3AA{:}S1{:}S2 \quad (6)$$

These reactions were performed at 10, 15, 20, and 25 °C.

RESULTS AND DISCUSSION

Evaluation of Experimental Errors. The accuracy of the method of obtaining thermodynamic parameters for the formation of TWJ complexes described in this work is dependent on knowing the concentrations of the reactants. Concentrations of individual oligonucleotides can be accurately determined by UV absorbance as described above. Reactions involving the addition of the third strand to form the TWJ are complicated by the fact that an equilibrium exists between the two strands in the cell (i.e., there will be some unbound oligonucleotides present as defined by K_B). Addition of the third strand, resulting in the formation of the more stable TWJ, will result in the formation of further two-strand complexes from the unbound strands. The K_B values for the reactions, however, were such that in all the reactions to form two-strand complexes less than 10% of the final concentration of oligonucleotides was unbound. The heats of association of the two-strand complexes are substantially less than the heats of formation of the TWJ, and thus, the potential errors from this phenomenon are not significantly greater than the errors inherent in the ITC technique (Connelly et al., 1990). This was demonstrated by the lack of concentration dependence of the enthalpies of binding observed and the fact that no biphasic titration curves were observed.

Two-Strand Reactions. A typical titration of single-stranded nucleotides is shown in Figure 2. This figure shows the titration of S3AA with S1 at 10 °C (eq 3b). The titration

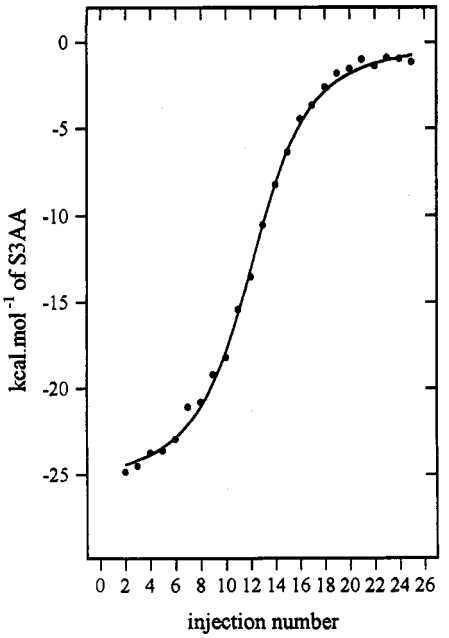

FIGURE 2: Isothermal titration calorimetry for the reaction of S3AA with S1 at 10 °C (eq 3b: S3AA + S1 = S1:S3AA). The titration was comprised of 25 10-μL injections of S3AA (160 μM) into the isothermal cell (1.39 mL) containing S1 (14.5 μM). Five minutes were allowed between injections for equilibration. The heats of dilution of both S3AA into buffer solution and buffer solution into S1 were assessed in separate titrations. The total heat of dilution of each injection for the reaction (2 μcal) was subtracted from the data shown. The solid line represents the best fit for variable parameters. Binding constant, K_B (1.7×10^6 M^{-1}), enthalpy of reaction, ΔH (–25.72 kcal mol^{-1}), and stoichiometry, N (1.01), were calculated according to published procedures (Wiseman et al., 1989).

consisted of 25 10-μL injections of S3AA (160 μM) into the isothermal cell containing S1 (14.5 μM). As the binding site(s) of the DNA in the cell was saturated, the heats of reaction were reduced until no further heat was observed (once the heats of dilution had been subtracted). The data resulted in the characteristic sigmoidal curve. Data were fit using methods previously described by Wiseman et al. (1989). Table 1 shows the data obtained for all the two-strand titrations performed according to eqs 1–3 in the temperature range 10–25 °C. The errors reported in Table 1 (as in all subsequent tables) are derived from the linear least-squares fits of the data from the individual titrations.

The stoichiometry parameter, N, obtained by fitting each of the two-strand ITC curves was found in all cases to approximate unity (mean value of $N = 1.04 \pm 0.04$). This finding agrees with the observations made by gel electrophoresis that S1, S2, and S3AA bind pairwise in 1:1 molar stoichiometry.

There appears to be no dependence on the order of addition of the strands in the titrations as shown for the reaction of S1 and S3AA. Table 1 contains the data measured for both eqs 3a and 3b. These values were found to be identical, within experimental error.

The binding constants calculated for the two-strand titrations from the ITC curves are shown in Table 1. The binding constants were found to decrease by approximately an order of magnitude as the temperature was increased from 10 to 25 °C. The enthalpies of binding showed a negative linear dependence on temperature as shown in Figure 3. From these data, ΔC_p for the two-strand reactions could be determined by linear least-squares fitting (as shown in Table 2). The heat capacity changes were large and negative varying from

Table 1: Experimental Data from Isothermal Calorimetric Titrations of Single Strands[a]

T (°C)	concentration (μM)		N	$K_B \times 10^{-6}$ (M^{-1})	ΔH (kcal mol^{-1})
	S1	S2			
10	735	60.0	1.01	0.30 ± 0.04	–10.70 ± 0.21
15	506	40.3	1.15	0.12 ± 0.01	–13.30 ● 0.40
20	509	52.0	1.03	0.08 ± 0.01	–14.25 ± 0.47
25	519	36.0	1.04	0.04 ± 0.01	–19.50 ± 1.99
	S3AA	S2			
10	600	52.2	1.04	1.70 ± 0.09	–15.35 ± 0.07
15	479	40.6	1.01	0.82 ± 0.05	–18.46 ● 0.16
15	479	50.9	1.09	0.83 ● 0.06	–18.61 ± 0.14
20	479	40.6	1.01	0.38 ± 0.02	–21.61 ± 0.24
25	600	52.2	1.02	0.16 ± 0.02	–24.47 ± 0.21
	S1	S3AA			
10	1470	60.0	1.02	1.90 ● 0.21	–24.57 ± 0.23
10	1470	30.0	1.05	1.70 ± 0.10	–25.96 ± 0.17
15	499	47.9	1.02	0.87 ± 0.07	–26.10 ± 0.14
20	499	47.9	1.07	0.59 ± 0.05	–26.37 ± 0.30
25	499	47.9	1.04	0.26 ● 0.02	–27.68 ± 0.32
	S3AA	S1			
10	160	14.5	1.01	1.70 ± 0.11	–25.72 ± 0.25
20	160	11.3	0.99	0.45 ± 0.50	–26.61 ± 1.05

[a] For each set of titrations, the oligonucleotide in the first column was titrated into the oligonucleotide in the second column, at the indicated temperature and concentrations. The parameters N, K_B, and ΔH were obtained by fitting each titration curve as previously described (Wiseman et al., 1989).

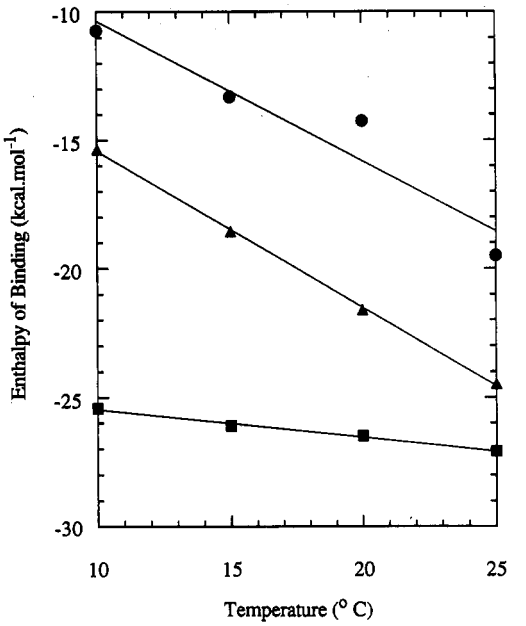

FIGURE 3: Plot of the enthalpy of binding, ΔH, versus temperature for the reaction of two strands. The data points are shown with linear least-squares fitting described by the following equations: (●) S2 + S1, $\Delta H = -0.55(T) + -4.89$, $R^2 = 0.91$; (▲) S2 + S3AA, $\Delta H = -0.61(T) + -9.34$, $R^2 = 0.99$; and (■) S1 + S3AA, $\Delta H = -0.13(T) + -24.39$, $R^2 = 0.99$.

–0.61 kcal mol^{-1} K^{-1} for S2:S3AA to –0.13 kcal mol^{-1} K^{-1} for S3AA:S1.

The linearity of the ΔH versus T plot (Figure 3) indicates that there are no additional contributions to enthalpy from, for example, temperature-dependent conformational changes. This fact is supported by gel electrophoretic studies (Leontis et al., 1991).

The base-pair sequences were designed to form a three-way junction in which each arm of the junction comprises six base pairs (Figure 1). The two-strand complexes should, therefore, contain five G-C interactions and one A-T interaction. Due

Table 2: ΔC_p Data for the Formation of Two-Strand and Three-Way Junction DNA Complexes[a]

reaction	ΔC_p (kcal mol^{-1} K^{-1})
S2 + S1 = S2:S1	−0.55 (±0.12)
S3AA + S2:S1 = S2:S1:S3AA	−1.07 (±0.20)
sum ΔC_p for TWJ	−1.62
ΔC_p obtained from ΔH_{1+2}	−1.59 (±0.15)
S3AA + S2 = S2:S3AA	−0.61 (±0.01)
S1 + S2:S3AA = S2:S3AA:S1	−0.90 (±0.34)
sum ΔC_p for TWJ	−1.51
ΔC_p obtained from ΔH_{1+2}	−1.56 (±0.17)
S1 + S3AA = S3AA:S1	−0.13 (±0.03)
S2 + S3AA:S1 = S3AA:S1:S2	−1.46 (±0.15)
sum ΔC_p for TWJ	−1.59
ΔC_p obtained from ΔH_{1+2}	−1.67 (±0.15)
Average ΔC_p for TWJ Assembly	
from sum of ΔC_p	−1.57
from ΔH_{1+2}	−1.61

[a] ΔC_p was calculated for the formation of the two-strand complexes S2:S1, S2:S3AA, and S3AA:S1 by linear least-squares fitting of the ΔH versus temperature data in Table 1. ΔC_p was calculated for the reactions of the two-strand complexes with the respective third strand to form the TWJ, S1:S2:S3AA, by linear least-squares fitting of the ΔH versus temperature data in Table 4. The overall value of ΔC_p for the formation of S1:S2:S3AA was calculated for each permutation of strand addition in two ways: by simply summing ΔC_p for each reaction and by linear least-squares fitting of the ΔH_{1+2} versus temperature data shown in Table 3.

to the similarity of binding interactions, the thermodynamic parameters associated with the base-pair interactions in the two-strand complexes alone might be expected to be similar. The two-strand titrations, however, showed pronounced differences in binding constants, enthalpy, entropy, and change in heat capacity. The binding constants for the formation of the S2:S1 complex at all temperatures were found to be significantly lower than for the other two reactions. The S1:S3AA duplex was found to be most stable. The difference in free energy of association between the most stable and least stable two-strand complex is approximately 1 kcal mol^{-1} throughout the temperature range studied (see the second column in Table 3).

The explanation for the significant differences in the thermodynamic parameters of formation of the two-strand complexes is not clear; however, it is likely to be the result of several contributing factors. The ordering and position of the base pairs are undoubtedly important. There is the potential to form additional interactions in the portions of the strands designed to be unbound. These would, however, require significant structural deformations. Native gel electrophoresis (Leontis et al., 1991) and UV melting experiments indicate that S2 may exist as a dimer. We observed no evidence of enthalpy of dissociation in the titrations to assess heats of dilution. In addition, the association of individual two-strand complexes to form dimers or higher oligomers may be possible. Again, we have no evidence for these types of structures, based on gel studies.

Formation of the TWJ Complex. The two-strand complexes were titrated with the corresponding third strand as described above to give the final TWJ product depicted in Figure 1. Addition of the third strand results in the formation of additional two duplex arms requiring 12 base-pair interactions. Reactions to form the TWJ complex were performed in all three permutations of the order of addition of the strands as given in eqs 4–6. A representative ITC curve for the reaction of a two-strand complex with the third strand is shown in Figure 4 (data were obtained at 20 °C for the reaction of S2 with S3AA:S1; eq 6). As described above, NMR data

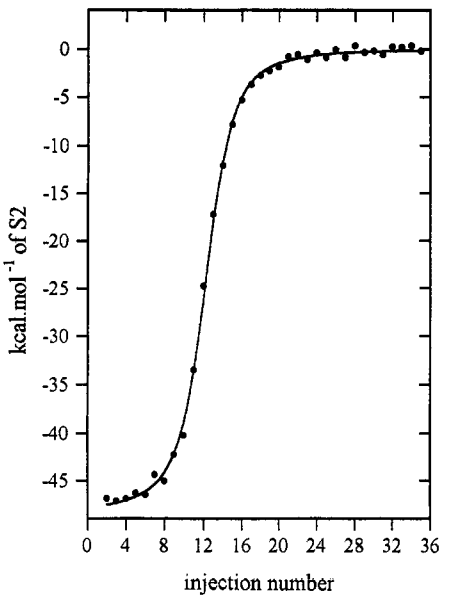

FIGURE 4: Isothermal titration calorimetry for the reaction of S2 with the complex of S3AA and S1 at 20 °C (eq 6: S2 + S3AA:S1 = S3AA:S1:S2). The titration was comprised of 35 7-μL injections of S2 (202 μM) into the isothermal cell (1.39 mL) containing the equimolar solution of S3AA and S1 (12.2 μM). Five minutes were allowed between injections for equilibration. The heats of dilution of both S2 into buffer solution and buffer solution into the complex were assessed in separate titrations. The total heat of dilution for each injection (7 μcal) was subtracted from the data shown. The solid line corresponds to the best fit for variable parameters. Binding constant, K_B (5.4 × 10^6 M^{-1}), enthalpy of reaction, ΔH (−48.39 kcal mol^{-1}), and stoichiometry, N (1.03), were calculated according to published procedures (Wiseman et al., 1989).

unequivocally show that all three arms of the TWJ are stable and fully based paired in the temperature range investigated in this work. NOESY spectra also show that there is no change in the normal B-type right-handed helical structure observed between 10 and 25 °C. UV melting experiments of the TWJ show no significant changes within the temperature range studied in this work (Thiviyanathan et al., manuscript in preparation).

All curves were fit to obtain the stoichiometry, N, the binding constant, K_B, and the enthalpy, ΔH, of reaction (as shown in Table 4). The stoichiometry of the associations of the third strand with the two-strand complexes is also reproducibly 1:1 (the mean value of N is 1.01 ± 0.03). The binding constants associated with these reactions are roughly an order of magnitude greater than observed for the two-strand interactions at any given temperature and also show decreasing values with increasing temperature. The ΔH values for these interactions are also greater than for the formation of the two-strand complexes as would be expected, based on the larger number of resulting interactions. Large negative ΔC_p values were obtained from the linear dependence of ΔH on temperature, as shown in Figure 5. They vary from −0.90 (±0.34) kcal mol^{-1} K^{-1} for S1 + S2:S3AA to −1.46 (±0.34) kcal mol^{-1} K^{-1} for S2 + S3AA:S1 (Table 2).

Once again, the linearity of the ΔH versus T plots emphasizes the fact that there are no further reactions contributing to the total enthalpy observed on binding of the strands. If there was some degree of melting of the two-strand substrates prior to addition of the third strand, the incremental heat associated with reforming these structures on formation of the TWJ would result in curvature of the plots.

Assuming that the contributions to the thermodynamics of TWJ formation of possible dimerization or other subsidiary

Table 3: Thermodynamic Parameters for the Formation of the Three-Way Junction S1:S2:S3AA in the Range 10–25 °C[a]

T (°C)	$\Delta G°_1$[b]	$\Delta G°_2$[c]	$\Delta G°_{1+2}$	ΔH_1	ΔH_2	ΔH_{1+2}	$T\Delta S°_1$	$T\Delta S°_2$	$T\Delta S°_{1+2}$
10	−7.09	−9.28	−16.37	−10.72	−48.63	−59.35	−3.63	−39.35	−42.98
15	−6.70	−9.08	−15.78	−13.30	−56.61	−69.91	−6.60	−47.53	−54.13
20	−6.58	−8.81	−15.39	−14.25	−60.39	−74.64	−7.67	−51.58	−59.25
25	−6.28	−8.62	−14.90	−19.50	−64.70	−84.20	−13.22	−56.08	−69.30
	$\Delta G°_1$[d]	$\Delta G°_2$[e]							
10	−8.07	−9.27	−17.34	−15.35	−47.52	−62.87	−7.28	−38.25	−45.53
15	−7.80	−8.97	−16.77	−18.54	−48.60	−67.14	−10.74	−39.63	−50.37
20	−7.48	−8.81	−16.29	−21.61	−55.25	−76.86	−14.13	−46.44	−60.57
25	−7.10	−8.53	−15.63	−24.47	−61.23	−85.70	−17.37	−52.70	−70.07
	$\Delta G°_1$[f]	$\Delta G°_2$[g]							
10	−8.10	−9.07	−17.17	−25.42	−32.90	−58.32	−17.32	−23.83	−41.15
15	−7.83	−9.26	−17.09	−26.10	−43.59	−69.69	−18.27	−34.33	−52.60
20	−7.67	−9.14	−16.81	−26.49	−48.81	−75.30	−18.82	−39.67	−58.49
25	−7.39	−8.87	−16.26	−27.08	−56.62	−84.30	−20.29	−47.75	−68.04

[a] ΔH values at each temperature are averages of the quantities tabulated in Table 1 (two-strand reactions) and Table 4 (three-strand reactions). $\Delta G°$ values were calculated from K_B for each experiment and averaged in those cases where more than one titration was carried out at the given temperature. Values of $T\Delta S°$ were calculated from $\Delta G°$ and ΔH. Values for $\Delta G°_{1+2}$ were calculated by summing $\Delta G°_1$ and $\Delta G°_2$ and likewise for ΔH_{1+2}. [b] 1 is S2 + S1. [c] 2 is S3AA + S2:S1. [d] 1 is S2+S3AA. [e] 2 is S1 + S2:S3AA. [f] 1 is S3AA + S1. [g] 2 is S2+S3AA:S1.

Table 4: Calorimetric Data for the Titrations of Single Strands into Double-Strand Complexes To Form the TWJ S1:S2:S3AA[a]

T (°C)	concentration (μM)		N	$K_B \times 10^{-6}$ (M^{-1})	ΔH (kcal mol^{-1})
	S3AA	S2:S1			
10	600	40.0	1.02	16.0 ● 0.2	−45.79 ± 0.24
10	160	13.2	1.00	13.0 ± 0.1	−51.47 ± 0.35
15	160	11.9	1.03	8.2 ± 0.70	−57.73 ± 0.45
15	192	10.4	1.00	7.3 ± 0.20	−55.48 ● 0.26
20	191	10.4	1.04	3.7 ± 0.60	−60.39 ± 0.32
25	319	26.5	0.98	2.1 ± 0.13	−64.70 ± 0.45
	S1	S2:S3AA			
10	735	45.4	1.01	9.7 ± 0.09	−44.40 ± 0.16
10	735	54.5	0.99	19.0 ± 10.00	−50.63 ± 0.87
15	170	5.0	1.06	6.4 ± 0.76	−48.60 ± 0.87
20	170	5.0	0.97	3.5 ± 0.32	−60.43 ± 1.25
20	170	16.0	1.04	3.9 ± 0.28	−50.06 ± 0.36
25	170	15.2	1.01	1.8 ± 0.20	−61.23 ± 0.41
	S2	S1:S3AA			
10	1045	81.8	0.95	10.0 ± 0.10	−32.90 ± 0.20
15	243	16.5	0.98	17.0 ± 0.20	−44.90 ± 0.28
15	202	12.2	0.99	6.6 ± 0.50	−44.13 ± 0.33
15	202	12.2	1.00	8.2 ± 0.70	−41.75 ± 0.31
20	243	16.5	1.02	7.6 ● 0.63	−49.23 ± 0.34
20	202	12.2	1.03	5.4 ± 0.29	−48.39 ± 0.27
25	243	16.5	1.00	3.2 ± 0.13	−56.62 ± 0.27

[a] This table provides the corresponding data for three-strand reactions that are provided in Table 1 for two-strand reactions.

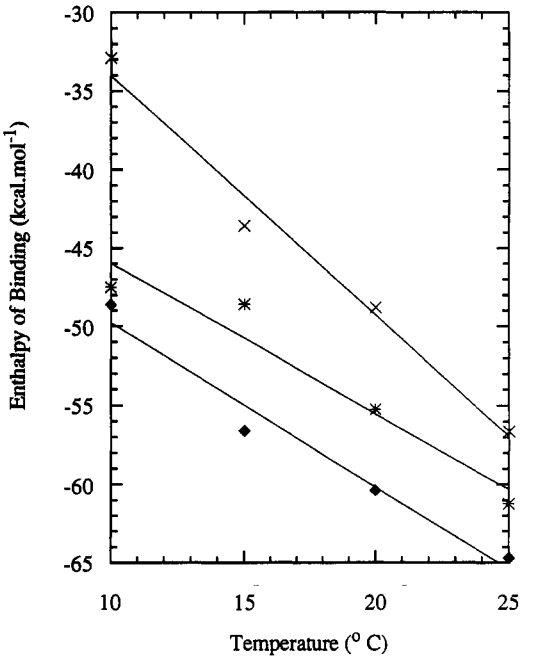

FIGURE 5: Plot of the enthalpy of binding, ΔH, versus temperature for the reaction of addition of the third strand to the two-strand complexes giving the TWJ product. The data points are shown with linear least-squares fitting described by the following equations: (◆) S3AA + S2:S1, $\Delta H = -1.07(T) + -38.96$, $R^2 = 0.90$; (*) S1 + S2:S3AA, $\Delta H = -0.90(T) + -37.64$, $R^2 = 0.66$; and (×) S2 + (S1:S3AA or S3AA:S1), $\Delta H = -1.46(T) + -20.19$, $R^2 = 0.96$.

reactions are not significant, the formation of the TWJ described in this work can be represented by the equation shown in Figure 6. Thus, the addition of the thermodynamic parameters (X) associated with the formation of the two-strand complexes to those involved in forming the final product gives the total values associated with TWJ formation:

$$\Delta X_1 + \Delta X_2 = \Delta X_{1+2} \quad (7)$$

These values are tabulated in Table 4. The thermodynamic cycle gives the total free energy associated with the formation of the TWJ as approximately −17.0 kcal mol^{-1} at 10 °C and −15.6 kcal mol^{-1} at 25 °C. The average enthalpy of formation of the TWJ complex ranges from −60 kcal mol^{-1} at 10 °C to −84.5 kcal mol^{-1} at 25 °C. These values are independent of the order of assembly of the three component oligonucleotide strands. The mean value of ΔC_p for TWJ formation is −1.57 (±0.06) kcal mol^{-1} K^{-1} (Table 2). The ΔC_P per base pair formed obtained in this work (−87 cal mol^{-1} K^{-1}) compares with that of Kallenbach and co-workers (−62 cal mol^{-1} K^{-1}; Lu et al., 1992).

We can estimate the free energy of formation of the junction itself from the three component duplex arms by comparing the sum of $\Delta G°_1$ for the formation of each of the arms with the average value of $\Delta G°_{1+2}$ (see Table 3). At 10 °C, the sum of the free energies of formation of the individual arms of the TWJ is −23.26 kcal mol^{-1}, whereas the average value of $\Delta G°_{1+2}$ is −16.96 kcal mol^{-1} (Table 3). The difference between these numbers, $\Delta G°_J$, is approximately 6 kcal mol^{-1}. At 25 °C, the $\Delta G°_J$ for the formation of the junction is 5 kcal mol^{-1}. These numbers give a rough estimate for the free energy of introduction of a three-way junction into a duplex region of DNA. They show that these reactions are thermodynamically unfavorable. This result is in agreement with competitive binding experiments in which radiolabeled S1 is mixed with

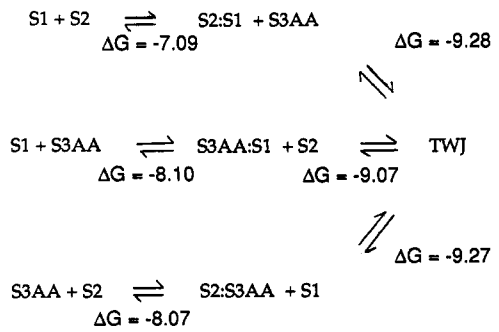

FIGURE 6: Schematic representation of the three permutations of reactions to give the final model TWJ product. The free energies of formation, $\Delta G°$, for the individual reactions are shown.

oligonucleotides S2 and S3AA as well as a third oligonucleotide, C1, which is complimentary to S1. An equilibrium is thereby established between the three-way junction complex S1:S2:S3AA and the duplex S1:C1. The equilibrium lies far to the side of the duplex S1:C1 as shown by gel electrophoresis (Leontis, unpublished results).

A theoretical value for the free energy of formation of the TWJ can be obtained using the theoretical approach of Doktycz et al. (1992). From the TWJ sequence of nucleotides in this work, the free energy of formation is −29.0 kcal mol^{-1} at 25 °C in 115 mM NaCl. This value corresponds to the average $\Delta G°_{1+2}$ value of −15.6 kcal mol^{-1} (Table 3). The reason for the large discrepancy is not clear; although as calculated from the component duplex arms (described in the previous paragraph), the free energy for the formation of the junction shows that this reaction is thermodynamically unfavorable ($\Delta G°_J$ = 13.4 kcal mol^{-1}). The theoretical methods of Breslauer et al. (1986) and Delcourt & Blake (1991) give calculated values of the free energy of formation of the TWJ of −24.2 and −27.5 kcal mol^{-1}, respectively; however, these were determined under very different buffer conditions to those used here.

The large negative ΔC_p obtained for the formation of the TWJ is characteristic of biomolecular binding interactions. Large negative ΔC_p values have been reported for protein–DNA interactions (Ha et al., 1989; Takeda et al., 1992; Spolar et al., 1994) and appear to correlate with the removal of hydrophobic binding surface area from solvent exposure. The additional contribution from the restriction in vibrational modes in molecules on forming interactions in the binding surface has also been intimated (Sturtevant, 1977; Ladbury et al., 1994). The determination of ΔC_p for oligonucleotide interactions has not been widely reported. In attempting to predict the structural implications of oligonucleotide reactions, understanding the determinants of this thermodynamic parameter will clearly be important.

NMR studies of the closely related TWJ complexes S1:S2:S3TT and S1:S2:S3TC have demonstrated that the unpaired bases are partly solvent exposed and that stacking of two of the three helices occurs across the junction along S1. These complexes differ from S1:S2:S3AA only in the nature of the unpaired bases on strand 3. The NMR data revealed that cytosine S1-C6 forms base pairs, in Watson–Crick fashion, with S3-G8 while S2-C7 forms base pairs with S2-G6. Furthermore, these two base pairs are stacked on one another (Leontis et al., 1993). NMR studies on another TWJ complex, having two unpaired cytosines, also showed unique stacking of two of the three helices and extrahelical conformations for the unpaired bases (Rosen & Patel, 1993). These studies have demonstrated unequivocally that TWJ complexes with two unpaired pyrimidines form structures with unique conformations. Base stacking is considered to be largely a hydrophobic interaction between neighboring bases. The large value of ΔC_p observed in the present experiments on S1:S2:S3AA could comply with the NMR observations if the hydrophobic stacking interactions replace solvent interactions.

This is the first report of ITC being used to obtain thermodynamic parameters for the formation of TWJs. The reaction results in a stable complex and is accompanied by a large negative heat capacity change. Detailed knowledge of three-dimensional structures and thermodynamics will increase our understanding of the forces which determine the stable conformations of nucleic acids and perhaps eventually allow this to be applied to the accurate prediction of structure from primary sequence information.

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